ISSN 1990-7478, Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology, 2009, Vol. 3, No. 3, pp. 245–253. © Pleiades Publishing, Ltd., 2009.
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Voltage-Gated Sodium Channels Are Targets for Toxins from the Venom of the Spider *Heriaeus melloteei*[1]

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Received March 27, 2009

Abstract—Three novel peptides were isolated from the venom of the spider *Heriaeus melloteei* (Thomisidae) and characterized. The peptides named Hm-1, 2 and 3 blocked voltage-gated Na^+ channels at concentrations in the order of 100 nM. Activity of the purified peptides was investigated in Na^+ channel isoforms of mammals and insects. Hm-1 and 2 appeared to act as pore blockers, whereas Hm-3 modulated the channel activation process. The toxins described exhibit minor similarity with other known peptides and may therefore constitute new groups of Na≅+ channel ligands.

Key words: spider venom, voltage-gated sodium channels, toxin, peptide structure, blocker, modulator

DOI: 10.1134/S1990747809030027

Voltage-gated sodium (Na^+) channels play a crucial role in processes of propagating action potentials in neuronal tissues and homeostasis maintenance [1]. Structure and function studies of these membrane transport systems play an important role in modern neurobiology. Indispensable tools for such studies are natural toxins specifically acting on these targets. In the 1970s–1980s, G.N. Mozhaeva with colleagues were exploring action mechanisms of some natural toxins, including plant alkaloid aconitine, steroid batrachotoxin (BTX) from the skin of Colombian frog, and polypeptide toxins from the scorpion *Buthus eupeus*, on various characteristics of Na^+ channels: conductance, selectivity, kinetics of activation and inactivation [2–7]. Since then, due to the emergence of new techniques and equipment, an arsenal of tools has been significantly enhanced, allowing detailed examination of pharmacology, structure-functional characteristics and physiological role of many types of Na^+ channels [8–12].

The spatial organization of Na^+ channels is still unclear. The α-subunit (~ 260 kDa) is the major channel component and represents an integral membrane protein containing all the functional elements of the channel: the ion-conducting pore, selectivity filter, voltage sensor, and different ligand binding sites. β-Subunits, for instance β1 (~ 38 kDa) and β2 (~ 33 kDa), are additional and play a modulatory role. The channel's α-subunit consists of four homologous domains (I–IV), each domain in turn consists of six transmembrane segments (S1–S6). The channel pore is formed between segments S5 and S6 of the four domains, which also contain the selectivity filter. It was found that the S4 segments play the role of the voltage sensor [13–15].

Using electrophysiological, biochemical, and molecular biological techniques at least nine isoforms of the voltage-gated Na^+ channel α-subunit ($Na_v1.1$–$Na_v1.9$) have been found in mammals and eight genes have been successfully expressed in a heterological system [15]. Currently, at least seven different ligand-binding sites (so-called receptor sites) of natural toxins and some pharmacological agents have been described in Na^+ channels. Two groups of toxins interact with receptor site 1: heterocyclic tetrodotoxin (TTX) and saxitoxin, as well as peptidic μ-conotoxins from venoms of marine snails of the genus *Conus*. In accordance with the sensitivity to TTX, Na^+ channel isoforms are allocated to the TTX-sensitive (TTX-S) and TTX-resistant (TTX-R: $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$). Toxins acting on site 1 do not alter the kinetics of the channels or their current-voltage relationships and influence only the ion conductivity, physically occluding the ion current, and thus are considered pore blockers. Other ligands modulate the activation and/or inactivation of the channels. Lipophylic grayanotoxin and alkaloids like veratridine, aconitine, and BTX bind to receptor site 2 and inhibit inactivation as well as shift the voltage-dependence of activation of the channels. Receptor site 3 is a target for a number of molecules: scorpion α-toxins, some marine shellfish, cone snail and spider toxins, all of which slow down or block the process of inactivation of the channels. Scorpion β-toxins as well as some spider toxins interact with site 4. These sub-

[1] The article was translated by the authors.

stances shift the threshold of the activation of the channels to the more negative membrane potentials. Brevetoxin and ciguatoxin potentiate Na^+ channels by targeting site 5; they shift the activation potential of the channels and also block the process of inactivation. δ-Conotoxins interact with the site 6 and slow down the inactivation of the channels. Site 7 was identified as the target of liposoluble pyrethroid insecticides and DDT, also inhibiting the channel inactivation [16–18]. In contrast to the wide variety of modulators, the set of the known Na^+ channel pore blockers is limited and includes only a few examples, among them certain spider toxins: hainantoxins I and III–V from *Haplopelma hainanum* [19–21], huwentoxins I and IV from *Haplopelma schmidti* [22–24], Tx 1 from *Phoneutria nigriventer* [25, 26], and Hm-1 and 2 from *Heriaeus melloteei* [27].

Despite certain success, structure and physiological role of many types of voltage-gated Na^+ channels remain unresolved; therefore, the problem of obtaining new modulators of their activity is highly relevant. By example of this work, we would like to show how using an arsenal of modern techniques, search of the new Na^+ channel blockers is performed.

EXPERIMENTAL

Biological material. Spider venoms were provided by A. Feodorov (Fauna Labs; Almaty, Kazakhstan). In every case, venoms were collected form 10–20 adult species of both genders by electrostimulation, frozen immediately and lyophilized.

Venom separation. Crude venom of the spider *Heriaeus melloteei* was separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Jupiter C_5 column (4.6 × 150mm, Phenomenex, USA) using a 60-min linear gradient of acetonitrile (0–60%, v/v) in aqueous trifluoroacetic acid (TFA; 0.1%, v/v) at a flow rate of 1 ml/min. Detection of eluate absorbance was performed at 210 and 280 nm. Fractions obtained were lyophilized and then tested. Active fractions were exposed to a second step HPLC on a Luna C_8 column (4.6 × 150 mm, Phenomenex, USA) using a 60-min linear gradient (30–70% or 10–50%) of elution solvent (50% acetonitrile, v/v, 20% isopropanol, v/v) in 0.1% TFA at a flow rate of 1 ml/min. The final stage of the active molecules purification was performed on a Luna C_{18} column (2 × 150 mm, Phenomenex, USA) using an 80-min linear gradient of acetonitrile (10–50%, v/v) in 0.1% TFA at a flow rate of 0.1 ml/min.

Insect toxicity assays. Venom fractions obtained were analyzed for toxicity using *Sarcophaga carnaria* larvae (mass of ~70 mg). Lyophilized substances were dissolved in physiological saline (140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$ and 5 mM HEPES, pH 7.2). Samples were injected using a microsyringe (Hamilton, USA) into the forth segment of larvae. The experiment was carried out in triplicate; pure saline injection served as control. Development of paralytic and lethal effects was monitored for 24 h.

Electrophysiological studies. For different Na^+ channel isoform expression in *Xenopus laevis* oocytes, cDNA encoding rat $rNa_v1.2$ and murine $mNa_v1.6$ were cloned into the plasmid pLCT1, whereas the gene encoding the rat isoform $rNa_v1.4$ was cloned into the plasmid pUI-2. For in vitro transcription these plasmids were linearized by the restriction enzyme NotI. The construct hβ1/pGEM-HE containing the gene of the human β1-subunit was linearized by the NheI enzyme. Plasmids containing cDNA of human isoform $hNa_v1.5$, human and rat β1-subunits (pSP64T), as well as rat $rNa_v1.8$ (pBSTA) were linearized using XbaI, EcoRI and NotI, respectively. Plasmids encoding insect channel subunits ($DmNa_v1$/pGH19-13-5 and tipE/pGH19) were linearized by NotI. Capped RNAs were synthesized from linearized plasmids using the mMESSAGE mMACHINE T7 transcription kit (Ambion, USA). *X. laevis* oocytes were harvested from the ovarian lobes of anaesthetized female frogs at the stages V–VI. Oocytes were injected with 50 nl of RNA (α : β-subunit ratio of 1:1) at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, USA). The oocytes were incubated for 2–4 days in solution that contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM HEPES (pH 7.4) and was supplemented with 50 mg/l gentamycin sulfate.

Active venom components were tested on the oocytes expressing genes of channel proteins using the two-electrode voltage clamp method at room temperature. Electrodes were filled with 3 M KCl, the resistance was <1 MΩ. Recorded data was analyzed using GeneClamp 500 (Axon Instruments, USA). In this study, we used the following protocols. In order to achieve a maximum activation of channels, the oocytes membrane depolarization was induced from the holding potential of –90 mV to 40 mV for 100 ms at a frequency of 0.2 Hz. The inhibitory effect was registered after addition of toxin as a reduction of the current amplitude. To study the current–voltage dependence of activation, membrane depolarization was induced from –90 mV to 40 mV at intervals of every 5 mV. Average Na^+ ion conductivity was calculated to build the activation curves; the obtained data were analyzed by the Boltzmann equation. Processing and visualization of the results was carried out using the program Origin (OriginLab, USA).

Mass spectrometry. Toxin molecular mass measurements were carried out using the method of matrix-assisted laser desorption-ionization (MALDI) on a M@LDI-LR mass spectrometer (Micromass, UK) with identification of positive ions in linear mode. The 2,5-dihydroxy benzoic acid (10 mg/ml in 50% (v/v) acetonitrile, 0.1% TFA) matrix was used. Samples were prepared using the dried droplet method: equal volumes (0.5 μl) of sample and matrix were mixed on the target

plate and left to dry in the air. Calibration was performed using the ProteoMass calibration kit with molecular mass range of 700–66000 Da (Sigma, USA).

Reduction of disulfide bonds and modification of thiol groups. Dried samples were dissolved in 40 μl of solution containing 6 M guanidine hydrochloride, 3 mM EDTA, 0.5 M Tris-HCl (pH 8.5). Then 2 μl of 1.4 M 1,4-dithiothreitol were added and the sample was incubated overnight at 30°C. Four μl of 50% 4-vinylpyridine in isopropanol (v/v) were added to the samples, followed by the incubation for 15–20 min at room temperature in the dark. Modified polypeptides were separated by RP-HPLC column on a Jupiter C_5 (2 × 150 mm, Phenomenex, USA) using a 60-min linear gradient of acetonitrile (15–60%) in 0.1% TFA at a flow rate of 0.3 ml/min.

CNBr cleavage. One nanomole of each pure alkylated peptide was dissolved in 20 μl of 80% TFA and 1 μl of 5 M CNBr in acetonitrile (Sigma, USA) was added. The samples were incubated for 18 h at room temperature in the dark. The reaction products were separated using RP-HPLC on a Jupiter C_5 column (2 × 150 mm).

Endoproteinase Glu-C cleavage. One nmol of each purified alkylated peptide was dissolved in 20 μl of 50 mM NH_4HCO_3 (pH 8) and 0.2 μg of endoproteinase Glu-C (Sigma, USA) were added. Probes were incubated for 4 h at 37°C. The reaction products were separated by RP-HPLC as described in the previous section.

Protein sequencing. The N-terminal amino acid sequences of the alkylated peptides were determined on a Procise Model 492 protein/peptide sequencer (Applied Biosystems, USA) according to the manufacturer's protocol.

Homology modeling. Models of the spatial structure of the novel peptides Hm-1, 2 and 3 were built using the publicly available program MODELLER 8v1 (*http://www.salilab.org/modeller*) on the basis of the known structure of the spider toxin agelenin (PDB code 2E2S, *http://www.rcsb.org/pdb*). Accuracy of the structure was checked using the program WHAT_CHECK (*http://swift.cmbi.kun.nl/gv/whatcheck*) with the further minimization of the molecular potential energy by the program TINKER (*http://dasher.wustl.edu/tinker*) in the force field CHARMM27.

RESULTS AND DISCUSSION

At the modern stage of the development of molecular-biological and physical-chemical methods, it becomes possible to identify and characterize active components from complex mixtures provided with very limited amounts of biological material. For example, representatives of spider families such as Thomisidae are characterized by a very small size (less than 10 mm) and, accordingly, we can expect only a few μl of their venom. With substances in orders of magnitude less than decades ago, we can not only assess the composition of the whole venom and perform preliminary tests on a variety of targets, but also run a detailed characterization of the active molecules. An in-depth study of the new substance mechanism of action can be performed on specific targets, for example, using a heterological gene expression system. With shortage of natural material or even its complete absence, genetic engineering approaches can help obtain polypeptide molecules in sufficient quantities for research. Such an approach is essential to investigate the peptide structure (computer modeling is an alternative) and identify functionally important residues. This work is an example of how, with modern equipment and well-planned strategy, search and detailed study of new molecules that act on voltage-gated Na^+ channels can be performed, operating with small quantities of the source material.

Object selection. At the first stage of this work, we tested venoms from 16 spider species belonging to nine different families[2] for inhibitory activity on voltage-gated Na^+ channels. Three isoforms of Na^+ channels were used as targets: TTX-S $Na_v1.2$, $Na_v1.7$ and TTX-R $Na_v1.5$; their genes were expressed in *X. laevis* oocytes. Test results are given in the table. It was found that the venoms of two spiders *H. melloteei* and *Misumena vatia* belonging to the family Thomisidae produce the most pronounced blocking effect. For further studies, the venom from the spider *H. melloteei* was selected as the most active.

Identification of the active components. Since insects are the natural prey of spiders, we estimated the insecticidal activity of the crude venom of *H. melloteei*. It was shown that the venom at doses of ≥15 mg/kg caused an immediate paralysis and death of the flesh fly (*S. carnaria*) larvae. The value of the minimal effective dose indicated presence of components highly toxic to insects. Quite often venom toxicity is based on components active on Na^+ channels, such as in the case of dangerous to humans Brazilian "wandering" spiders of the genus *Phoneutria* and Australian "funnel-web" spiders of the genera *Atrax* and *Hadronyche* [28, 29].

With the aim of purification of active molecules, the strategy of venom multi-step HPLC separation was suggested (Fig. 1). All the fractions collected during the separation were tested for the presence of the required activity. Figure 1a shows the first stage of venom separation. Blocking activity towards mammalian Na^+ channels (isoforms $Na_v1.2$ and $Na_v1.5$) was found in fractions 3–6, whereas fractions 2–6 possessed insecticidal activity. Thus, the activity profiles overlap, which may be related to the effect of the same compounds. According to the MALDI mass-spectrometry, active fractions contained components with molecular masses in the range of 3–10 kDa, which corresponds to the most frequently encountered masses of spider venom

[2] Classified by A. Feodorov (Fauna Labs; Almaty, Kazakhstan).

Crude spider venom activity tests

Families	Species	$Na_v1.2$	$Na_v1.5$	$Na_v1.7$
Agelenidae	*Agelena orientalis*	9	3	9
Eresidae	*Eresus* sp.	–	34	17
	Stegodyphus sp.	26	52	–
Gnaphosidae	*Drassodes* sp.	33	71	35
Lycosidae	*Alopecosa* sp.	†	†	†
	Desertosa sp.	†	†	†
	Geolycosa sp.	†	†	†
	Hippocosa sp.	†	†	†
	Lycosa singoriensis	†	†	†
	Nenilinia sp.	†	†	†
Miturgidae	*Cheiracanthium punctorium*	†	†	†
Philodromidae	*Tibellus oblongus*	47	53	9
Theridiidae	*Latrodectus dahli*	6	36	8
Thomisidae	*Heriaeus melloteei*	87	81	80
	Misumena vatia	69	36	100
Zodariidae	*Lachesana tarabaevi*	†	†	†

Note: The values represent the percent of Na^+ current amplitude decrease in various isoforms at a venom concentration of 0.1 mg/ml. -, not tested; †, oocyte lysis.

peptides. Fractions 3, 5 and 6 have been selected for further investigation; we also plan to study the active components of fractions 2 and 4 in the future. As a result of two additional stages of HPLC, Na^+ channel blockers named Hm-1, 2 and 3 were purified to homogeneity (Fig. 1). Purity of the isolated compounds was confirmed by analytical HPLC and MALDI mass-spectrometry. The measured average molecular masses of the toxins are 4171.9 Da (Hm-1), 4555.3 Da (Hm-2) and 3907.7 Da (Hm-3).

Structure of the new blockers. Full amino acid sequences of the new Na^+ channel blockers (Fig. 2) were established by a combination of automated Edman degradation, selective cleavage of polypeptide chains at methionine (with CNBr) and glutamic acid residues (with endoproteinase Glu-C) and mass-spectrometry. Because of the difference of measured molecular masses and the corresponding calculated values based on the sequence (Δ = 1 Da), we concluded that toxin Hm-1 carries a posttranslational modification, namely, C-terminal amidation. All new peptides contain six cysteine residues forming three intramolecular S–S bridges. Location of cysteine residues in the amino acid sequences suggests the formation of the "cystine knot" spatial motif characteristic of many other spider peptide toxins. For Hm-2 and 3, no similar sequences were found in the UniProt database (*http://www.uniprot.org*), but they showed some degree of similarity between each other (46% identical residues). Hm-1 sequence showed a low degree of similarity with the known peptides agelenin (UniProt code P31328) and μ-agatoxin-2 (P11058) isolated from spider venoms and active on voltage-gated Ca^{2+}- and Na^+ channels, respectively (Fig. 3).

The spatial structure of the isolated molecules is not yet established. However, with the help of computer modeling it is possible to build their model structures on the basis of similarities with the toxins for which the 3D structure has already been resolved. Figure 4 presents the results of homology modeling of Hm-1, 2 and 3 on the template structure of agelenin, a toxin acting on Ca^{2+} channels that has the most similar amino acid sequence to the newly described peptides and is characterized by the "cystine knot" fold [30]. In the future we plan to identify amino acid residues responsible for interaction with the channel protein, and their spatial location on the models will indicate the possible pharmacophore.

Functional characterization. Application of all studied compounds at concentrations ~100 nM on Na^+ channels decrease the amplitude of the Na^+ current (Figs. 5, 6). In the case of Hm-1 and 2, no changes in

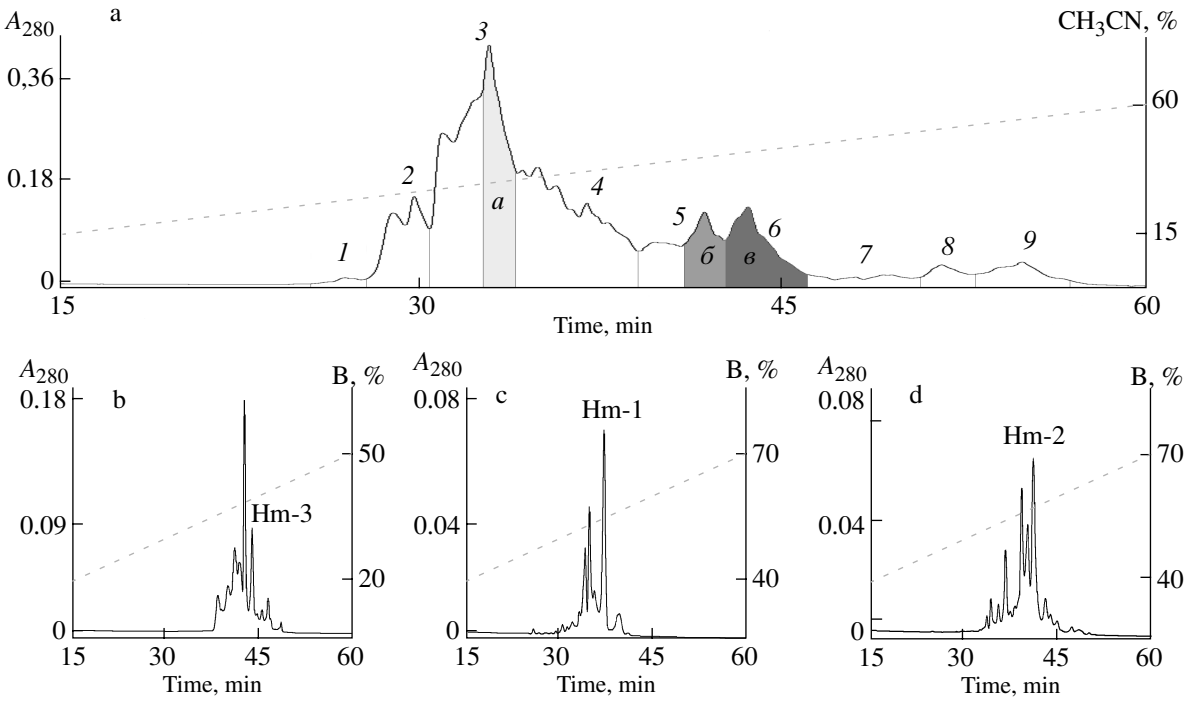

Fig. 1. Isolation of Na^+ channel blockers from the venom of *H. melloteei*. (a) Separation of 20 µl of crude venom by RP-HPLC on a Jupiter C_5 (4.6 × 150 mm) column using a 60-min linear gradient of acetonitrile (indicated with a line) at a flow rate of 1 ml/min. Numbers indicate fractions tested for insecticidal activity. The second step of separation of the active fractions *a* (b), *b* (c), and *c* (d) on a Luna C_8 (4.6 × 150 ÏÌ) column using a linear gradient of solvent B (50% acetonitrile, 20% isopropanol; marked by line) at a flow rate of 1 ml/min. The active fractions are indicated as Hm-1, Hm-2 and Hm-3.

the current–voltage relationships of the channels, as well as the kinetics of activation and inactivation occur (Fig. 6) [27]. Thus, the mode of action of these toxins appears to be pore-blocking. For all the new molecules no significant specificity of action with respect to isoforms of mammalian Na^+ channels was found. Figure 5 shows recordings of Na^+ currents modified by applications of the toxin Hm-2. It is seen that this peptide is most active on the insect channels, as well as mammalian TTX-S $Na_v1.2$ and $Na_v1.4$, and least active on the TTX-R $Na_v1.5$ and $Na_v1.8$. For the muscle-type channel (isoform $Na_v1.4$), the dose–response curves were obtained, and the effective concentrations causing a 50% reduction in the amplitude of Na^+ current were

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Hm-1 GCIPYGKTCEFWSGPWCCAGK-CKLNVWSMTLSCTRNF-NH2     37
Hm-2 GCIPSFGECAWFSGESCCTGI-CKWVFFTSKFMCRRVWGKD-OH 40
Hm-3 GCIAKNKECAWFSGEWCCGALSCKYSIKRNLKICV-OH        35
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Fig. 2. Amino acid sequences of the isolated Na^+ channel blockers. Methionine and glutamic acid residues are in bold, cysteine residues are shaded grey. The number of amino acid residues in the polypeptide chain of the corresponding toxin is indicated in the right column. Gaps were introduced to optimize the sequences comparison.

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Hm-1          -GCIPYGKTCEFWSGPWCCAG-KCKLNVWSMTLSCTRNF
ageленин      GGCLPHNRFCNALSGPRCCSGLKCKELSIWDSR-CL--- 40
μ–агатоксин 2 -ECATKNKRCADWAGPWCCDGLYCSCRSYPGCM-CRPSS 32
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Fig. 3. Hm-1 amino acid sequence comparison with other known peptides. Identical residues are shaded grey. The percent of identical amino acid residues with Hm-1 is indicated in the right column.

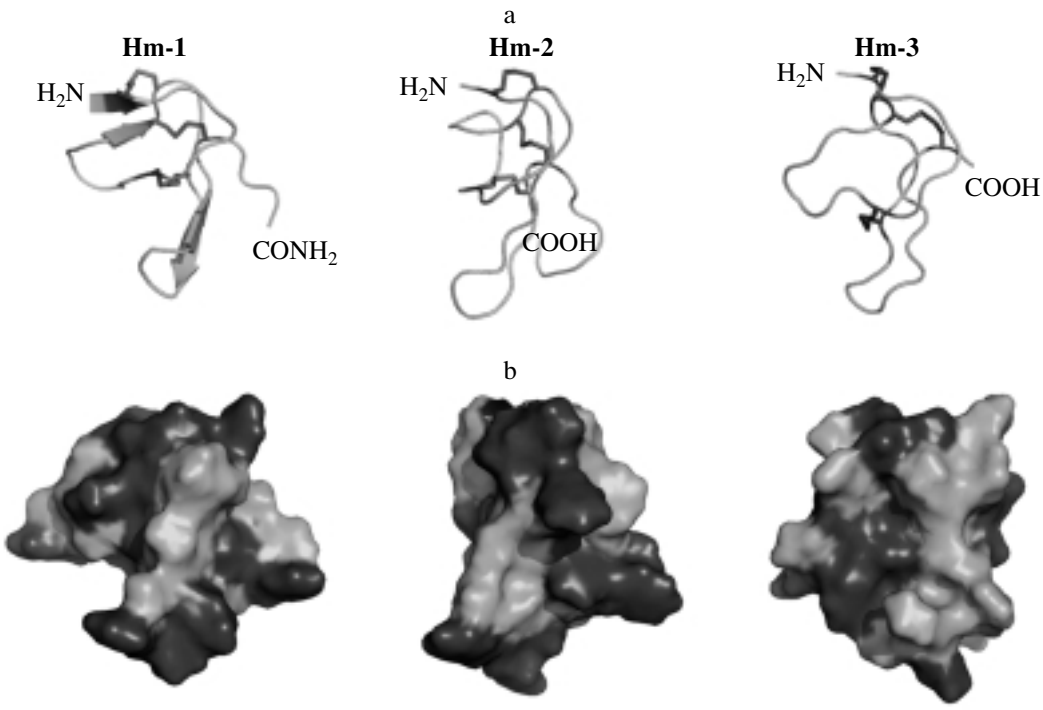

Fig. 4. Spatial model structures of Hm-1, 2 and 3. (a) Ribbon models. Cysteine residues and disulfide bonds are marked dark grey. N- and C- termini are indicated. (b) Space-filling models. Hydrophobic residues are marked dark gray and hydrophilic ones, light gray.

identified, which amounted to ~350 nM and ~160 nM in case of Hm-1 and 2, respectively [27].

Values of the effective concentrations for the described peptides are sufficiently high in comparison with some other toxins but the uniqueness of Hm-1 and 2 is that, unlike the vast majority of polypeptide ligands of Na^+ channels, they appear to be typical pore blockers. This feature brings them together with TTX and saxitoxin, and therefore they are expected to interact with the same parts of the channel, i.e., receptor site 1. Hm-3, despite considerable similarity in amino acid sequence with Hm-2 (46% identical residues), has a different mechanism of action. In Fig. 6, a distinct shift in current-voltage relationship of the channel under the action of Hm-3 is noticeable, whereas such an effect is absent in the case of Hm-1 and 2. Similar mode of action was found for some other spider toxins: JZTX-III from the spider *Chilobrachys jingzhao* and ProTx-I and II from the spider *Thrixopelma pruriens* that bind to receptor site 4 [31, 32]. We assume that Hm-3 also interacts with site 4. It should be noted that the classical Na^+ channel modulators acting on site 4 are the scorpion β-toxins. The mode of their action, however, is different: the activation process is not inhibited but instead potentiated, the threshold potential is shifted to the more negative values [33]. The same effects are seen with μ-agatoxins from the spider *Agelenopsis aperta* and Magi 5 from the spider *Macrothele gigas* [34, 35]. δ-Palutoxins from the spider *Paracoelotes luctuosus* interact with site 4, but the physiological effects are similar to those of scorpion α-toxins [36]. Thus, there is a wide variety in pharmacological activity of Na^+ channel peptide modulators. Peptides that interact with common sites of the channel protein can have different effects on its activity and conversely, peptides that interact with different receptor sites may produce similar physiological effects.

CONCLUSIONS

As a result of this work, we have described three polypeptide molecules from the venom of the spider *H. melloteei* that act on Na^+ channels. The low degree of similarity with other known peptides suggests that these substances belong to new groups of Na^+ channel blockers. In doing so, Hm-1 and 2, it seems, are pore blockers and are associated with receptor site 1, and Hm-3 interferes with channel activation and is likely to interact with receptor site 4. Molecules described in this paper represent new tools for studying the structure and function of Na^+ channels.

ACKNOWLEDGEMENTS

We are grateful to A.L. Goldin (University of California, Irvine, USA), G. Mandel (Stony Brook University, Stony Brook, USA), R.G. Kallen (University of Pennsylvania, Philadelphia, USA), J.N. Wood (University College, London, UK), S.H. Heinemann

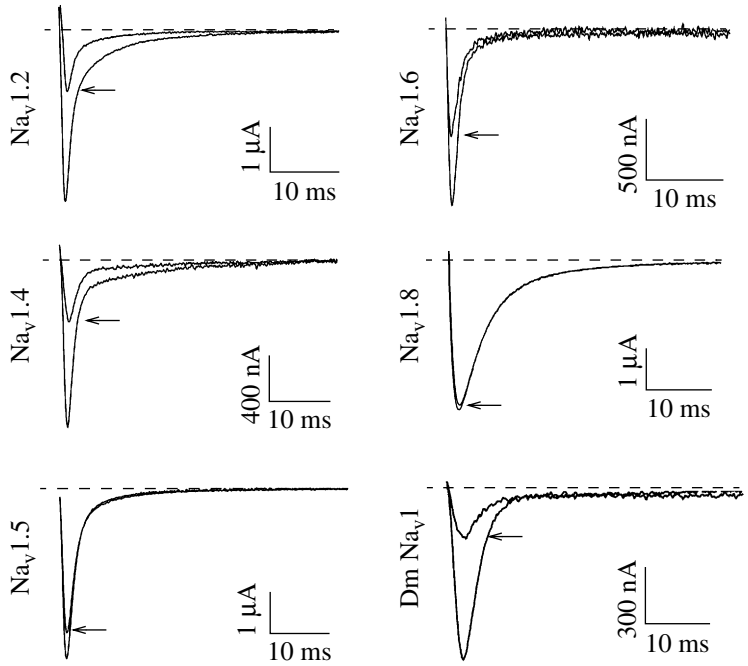

Fig. 5. Effects of Hm-2 on different Na^+ channel isoforms: $Na_v1.2$, 1.4, 1.5, 1.8 and $DmNa_v1$ expressed in *Xenopus* oocytes. The arrow indicates the Na^+ current peak amplitude in the presence of 200 nM of toxin.

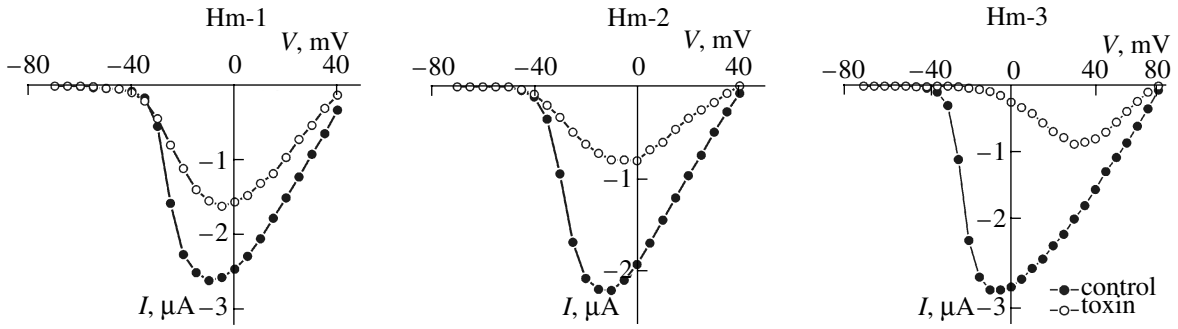

Fig. 6. Current–voltage relationships of Na^+ channel muscle isoform (Na_v 1.4) in the presence of toxins. Filled circles (@), control; open circles (@), toxin applications. Concentrations of toxins: Hm-1, 200 nM; Hm-2, 200 nM; Hm-3, 1 μM.

(Friedrich-Schiller-Universität, Jena, Germany), and S.C. Cannon (University of Texas, Dallas, USA), M.S. Williamson (IACR-Rothamsted, UK) for sharing plasmids with genes encoding Na^+ channels. We also thank S. Debaveye for her technical assistance in molecular biology.

The work was supported by the Russian Foundation for Basic Research, the Program of Cell and Molecular Biology of Russian Academy of Sciences, BIL05/50 (Bilateral collaboration between Flanders and the Russian Federation), G.0257.08 (F.W.O. Vlaanderen), OT-05-64 (K.U. Leuven), UA P6/31 (Interuniversity Attraction Poles Program – Belgian State – Belgian Science Policy).

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